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## **Cordycepin (3'-Deoxyadenosine), an Inhibitor of mRNA Polyadenylation, Suppresses Proliferation and Activates Apoptosis in Human Epithelial Endometriotic Cells in vitro**

Imesch, P ; Hornung, R ; Fink, D ; Fedier, A

**Abstract:** Background/Aims: Endometriosis is a benign but chronic disorder associated with pelvic pain and infertility. Enhanced proliferation and reduced apoptosis susceptibility are characteristics of endometriosis. Cordycepin is a poly(A) polymerase inhibitor. It induces shortening of poly(A) tails, leading to destabilization of mRNAs and finally to proliferation inhibition and cell death in normal and tumor cells. The potential of cordycepin to block proliferation and survival of 11z human immortalized epithelial endometriotic cells was determined. Methods: 11z cell cultures were treated with cordycepin. Cordycepin-induced inhibition of proliferation and alterations in protein expression and protein phosphorylation were determined by the methyl thiazolyl tetrazolium assay and immunoblot analysis, respectively. Results: Cordycepin induced the rapid and significant upregulation of the cell cycle progression inhibitor p21 and the downregulation of the cell cycle progression promoter cyclin D(1), finally leading to the inhibition of the proliferation of 11z human epithelial endometriotic cells. Cordycepin reduced the phosphorylation of the p38 mitogen-activated protein kinase and the retinoblastoma protein. It also activated caspase-dependent, intrinsic apoptosis, as documented by the proteolytic cleavage of the caspase-9, caspase-3 and the poly(ADP ribose) polymerase 1 precursor. Conclusion: The mRNA polyadenylation inhibitor cordycepin inhibits proliferation and survival of endometriotic cells.

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**Cordycepin (3'-deoxyadenosine), an Inhibitor of mRNA Polyadenylation, Suppresses Proliferation, and Activates Apoptosis in Human Epithelial Endometriotic Cells *in vitro***

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**Short title:** Effect of Cordycepin in endometriotic cells

**Key words:** Endometriosis, Proliferation inhibition, Apoptosis, Cordycepin, mRNA polyadenylation

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## **Abstract**

**Background/Aims:** Endometriosis is a benign but chronic disorder associated with pelvic pain and infertility. Enhanced proliferation and reduced apoptosis susceptibility are characteristics of endometriosis. Cordycepin is a poly(A) polymerase (PAP) inhibitor. It produces shortening of poly(A) tails, leading to de-stabilization of mRNAs, and finally to proliferation inhibition and cell death in normal and tumor cells. The potential of Cordycepin to block proliferation and survival of 11z human immortalized epithelial endometriotic cells was determined.

**Methods:** 11z cell cultures were treated with Cordycepin. Cordycepin-induced inhibition of proliferation and alterations in protein expression and protein phosphorylation were determined by the MTT-assay and immunoblot analysis, respectively.

**Results:** Cordycepin induced the rapid and significant upregulation of the cell cycle progression inhibitor p21 and the downregulation of the cell cycle progression promoter cyclin D1, finally leading to the inhibition of the proliferation of 11z human epithelial endometriotic cells. Cordycepin reduced the phosphorylation of the p38 MAPK and the retinoblastoma protein (pRb). It also activated caspase-dependent, intrinsic apoptosis, as documented by the proteolytic cleavage of the caspase-9, caspase-3, and the PARP-1 precursor.

**Conclusion:** The mRNA polyadenylation inhibitor Cordycepin inhibits proliferation and survival of endometriotic cells.

## Introduction

Endometriosis is a common, benign, and chronic disorder characterized by the proliferation of endometrial tissue outside the uterine cavity. Endometriosis is frequently associated with infertility and pelvic pain in affected women. The prevalence of endometriosis reaches 10-15% in women in the reproductive age and incidence rates can reach 30-50% among women with infertility. Endometriosis is not a life-threatening disease, but it causes a substantial negative impact on physical, psychological and social integrity of affected women. These include well-being, personal relationships, and desire for children. It also causes time off work and the need for expensive surgery and medical therapies. Diagnosis is difficult and needs an invasive approach. Available treatments (surgical and medical) are symptomatic rather than causative, because etiology and pathophysiology are still not completely understood. Endometriosis is an estrogen-dependent disease and standard medical treatment aims at downregulating ovarian function and/or antagonizing the effect of estrogen in ectopic endometrial implants using GnRH-Analoga, progestins, and contraceptives. Recent research has focused on inhibitors of angiogenesis and matrix metalloproteinases and on epigenetically acting agents [1-4].

Cordycepin (3'-deoxyadenosine), the main constituent of the mycelia of *Cordyceps militaris*, has numerous biological activities, including inhibition of cell proliferation, activation of apoptosis, and inhibition of cell migration and invasiveness [5-10]. Cordycepin has been shown to reduce tumor formation in mice [5] and has therefore been proposed as cancer drug. Cordycepin is an adenosine analogue and inhibits mRNA polyadenylation, presumably by causing chain termination after it has been incorporated as cordycepin-triphosphate [11]. Polyadenylation of mRNA, also referred to as 3'-end mRNA processing, is crucial to mRNA stability and to nuclear export of mRNA [12]. The stabilization of the newly transcribed mRNA and the translation of mRNA into a protein are crucial steps in protein synthesis in all cells. mRNA polyadenylation is a regulated process carried out in a multiprotein-complex,

wherein poly(A) polymerase (PAP) is the enzyme performing the addition of multiple adenosine-monophosphates (150 to 250) to the mRNA, resulting in a poly(A) tail. Cordycepin, lacking the 3'-OH-group normally present in adenosine, leads to the shorting of poly(A) tail length in a dose-dependent manner [13], confirming its PAP-inhibitor activity. PAP has been suggested as potential therapeutic target [14] and its enzymatic activity even as potential independent prognostic marker in primary breast cancer [15].

Because enhanced proliferation and reduced apoptosis susceptibility are characteristics in endometriosis, we determined the effects of Cordycepin on proliferation and apoptosis in 11z human immortalized epithelial endometriotic cells.

## Materials and Methods

### *Cell culture and drug*

The immortalized human epithelial endometriotic cell line (11z) employed in this study was provided by Dr. A. Starzinski-Powitz (Institute of Anthropology and Human Genetics, Johann-Wolfgang-Goethe University, Frankfurt, Germany). It was generated by *in situ* electroporation of primary human peritoneal epithelial endometriotic cells with SV-40 T-antigen. The characteristics of this cell line have been previously described [16, 17]. 11z cells were cultured in DMEM medium (21980; Invitrogen, Basel, Switzerland) containing 10% FCS (Oxoid, Basel, Switzerland), penicilin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere with 10% CO<sub>2</sub> and 95% humidity. Cordycepin (3'-deoxyadenosine) was purchased (Sigma, Buchs, Switzerland) and aliquots prepared in water were stored at -20°C.

### *Proliferation inhibition*

Proliferation inhibition of 11z cells in response to Cordycepin was assessed by the MTT-assay. Briefly, 30'000 cells in 200 µl medium were seeded into 96-well plates. Three days after seeding cultures were left untreated (controls) or were treated with various concentrations of Cordycepin for 96h. MTT-dye (dissolved in PBS) was added to a final concentration of 500 µg/ml. After 4h, the medium was removed and the crystals were dissolved in 200 µl DMSO. Optical density (OD, absorbance at 540 nm) was measured (SpectraFluor Plus Reader; TECAN AG, Hombrechtikon, Switzerland). Data are presented as the proliferation relative to untreated control (calculated from the respective OD values) as a function of Cordycepin concentration. Data points are the mean  $\pm$  SD of three independent experiments performed in triplicates.

### *Preparation of cell lysates and immunoblot analysis*

Cell lysates were produced from 11z cell cultures that were subconfluent at the time of analysis (avoiding undesired effects due to factors like contact inhibition). Cells were grown to 70% confluence, treated with various concentrations of Cordycepin for various periods of time as indicated, and lysed for immunoblot analysis performed following standard protocols. Protein concentration of cell lysates was determined by the BCA Protein Assay Kit (23227; Pierce, Perbio Science, Lausanne, Switzerland). Twenty µg cell lysate protein was loaded and separated using SDS-PAGE, followed by blotting onto a polyvinylidene difluoride membrane (Amersham Biosciences, Otelfingen, Switzerland). Proteins were detected by the specific primary antibodies and the respective secondary, horseradish peroxidase-conjugated anti-mouse (M15345; Transduction Laboratories, Lexington, KY) or horseradish peroxidase-conjugated anti-rabbit (7074; Cell Signaling; BioConcept, Allschwil, Switzerland) antibodies. The primary antibodies used were: p21 (2946; Cell Signaling), cyclin D1 (2926; Cell Signaling), PARP-1 (9542; Cell Signaling, recognizing both the 116 kD full-length PAPR-1

and the cleaved 89 kD fragment), caspase-9 (9502; Cell Signaling, recognizing the 47 kD precursor and the 37 kD and 17 kD fragments), caspase-3 (9662; Cell Signaling, recognizing the 37 kD precursor and the 17 kD fragment), phospho-ERK1/2 (9106; Cell Signaling, recognizing ERK phosphorylated at thr202 and tyr204), ERK1/2 (9102, Cell Signaling, recognizing both the non-phosphorylated and the phosphorylated ERK1/2), phospho-p38 (9211; Cell Signaling, recognizing p38 MAPK phosphorylated at thr180 and tyr182), p38 (9212; Cell Signaling, recognizing both phosphorylated and non-phosphorylated p38 MAPK), and phospho-pRb (9308; Cell Signaling, recognizing Rb protein phosphorylated at ser807 and ser811). Mouse anti- $\beta$ -actin (A5441; Sigma) was used as sample loading control. Complexes were visualized by enhanced chemiluminescence (Amersham Biosciences) and autoradiography. Quantitative analysis of the complexes (intensity on the autoradiograph) was performed by densitometry (normalized against  $\beta$ -actin) using the Scion Image 4.01 Win software (Scion Corporation, Frederick, MD).

### *Statistical analysis*

Mean  $\pm$  SD values were calculated (where appropriate). Statistical analysis was performed using the two-tailed Student's t-test.  $p$  values  $<0.05$  are considered statistically significant.

## **Results**

### *Cordycepin inhibits proliferation of 11z cells*

Endometriosis is characterized by increased proliferation of endometriotic tissue. We determined whether Cordycepin inhibits proliferation of 11z cells. MTT-assay data showed that Cordycepin significantly inhibited the proliferation of these cells in a concentration-

dependent manner (**fig. 1**). Half-maximal inhibition ( $IC_{50}$ ) was reached with 490  $\mu$ M Cordycepin (96h treatment). Proliferation of 11z cells is therefore inhibited by Cordycepin.

*Cordycepin upregulates p21 and downregulates cyclin D1 expression and pRb phosphorylation*

p21 is an endogenous cell cycle progression inhibitor which is upregulated in cancer cells in response to cytostatic and cytotoxic agents. We determined whether Cordycepin induces upregulation of p21 expression in 11z cells. Immunoblot data demonstrated (**fig. 2A**) that Cordycepin produced a rapid (14h of Cordycepin treatment), concentration-dependent upregulation of p21, which was detectable already with 50  $\mu$ M Cordycepin. Cordycepin also produced in a time- and concentration-dependent manner a strong decrease in the levels of cyclin D1 (**fig. 2B**) and of phosphorylated (serines 807 and 811) retinoblastoma protein (pRb) (**fig. 2C**) within 48h of treatment, two positive regulators of cell cycle progression. Proliferation inhibition by Cordycepin is thus accompanied by upregulation of p21 and downregulation of cyclin D1 expression and pRb phosphorylation.

*Cordycepin inhibits phosphorylation of p38 MAPK*

The extracellular signal-regulated kinase (ERK1/2) and the p38 mitogen-activated protein kinase (MAPK) are important signal transduction pathway protein kinases and believed to have also a role in endometriosis [18]. We determined the effects of Cordycepin on the phosphorylation of the ERK1/2 and the p38 MAPK, a characteristic of the enzymatic activity of these kinases in 11z cells. Immunoblot (**fig. 3A**) and densitometry (**fig. 3B**) demonstrated that Cordycepin produced a detectable (48h of treatment) and then substantial (72h of treatment) decrease of phosphorylation of p38 MAPK (threonine 180 and tyrosine 182) in a concentration- and time-dependent manner. A decrease in ERK1/2 phosphorylation (threonine



202 and tyrosine 204) was only detected after 96h with the highest (600  $\mu$ M) Cordycepin concentration. Proliferation inhibition by Cordycepin is therefore accompanied by downregulation of p38 MAPK signaling.

#### *Cordycepin activates caspase-dependent apoptosis*

Resistance to apoptosis is one of the characteristics of endometriosis. We determined whether Cordycepin, like in cancer cells [7], activates apoptosis in 11z cells. Immunoblot data (**fig. 4**) demonstrated proteolytic cleavage of the 116 kD PARP-1 precursor into a 89 kD-fragment (a measure for ongoing apoptosis) with 600  $\mu$ M Cordycepin after 48h of treatment, with 500  $\mu$ M Cordycepin after 72h of treatment, and with 400  $\mu$ M Cordycepin after 96h of treatment. Cordycepin also produced detectable decreases in the levels of the 47 kD caspase-9 and the 37 kD caspase-3 precursors after 48h of treatment and strong decreases after 72h and 96h of treatment, respectively. The respective cleaved fragments were not detected. Cordycepin thus activates caspase-9- and caspase-3-dependent apoptosis in 11z cells.

## **Discussion**

mRNA polyadenylation plays essential roles in mRNA stability, nuclear export, and translation and is an important step in many cellular processes [12]. Abrogation of mRNA polyadenylation through PAP inhibition has been shown to associate with anticancer activities [5]. PAP has therefore been proposed as therapeutical target and inhibitors mRNA polyadenylation as potential anticancer agents.

Endometriosis is characterized by increased proliferation, reduced apoptosis susceptibility, and angiogenic and invasive potential and therefore shares common features

with cancers. We hypothesized that inhibition of mRNA polyadenylation by the PAP inhibitor Cordycepin negatively affects proliferation and survival in 11z epithelial endometriotic cells *in vitro*. We demonstrate that proliferation can be inhibited and apoptosis can be activated by Cordycepin in these cells. This study is the first to address the issue of encountering these cellular processes crucial to endometriosis by the inhibition of mRNA polyadenylation. We conclude (i) that human epithelial endometriotic cells are sensitive to mRNA polyadenylation inhibition and (ii) that Cordycepin might be a potential candidate for treatment of endometriosis.

Increased proliferation is an important characteristic in endometriosis and regulators of cell cycle progression are targets for proliferation suppression. Proliferation and cell cycle progression are highly regulated processes involving the concerted action of cell cycle promoters (e.g. cyclins) and cell cycle inhibitors (e.g. p21, pRb). Cyclins complex with cyclin-dependent kinases (CDKs) at a specific point during G1 and phosphorylate and inactivate pRb, allowing cell cycle progression. p21 is an stress-responsive inhibitor of cell cycle progression and thus a crucial factor in the inhibition of proliferation. p21 interacts and forms heterodimeric complexes with cyclin-dependent kinases with cyclins, promoters of cell cycle progression and proliferation [19]. As an inhibitor of CDKs, p21 prevents phosphorylation of pRb, thereby repressing the transcription of genes required for the G1-S transition of the cell cycle and thus inhibiting proliferation [20-22]. Hypophosphorylated pRb acts as a tumor suppressor that may have a role in endometriosis [23]. Our results show that Cordycepin upregulates p21 expression and downregulates expression of cyclin D1 and phosphorylation of pRb, suggesting that these events contribute to the proliferation inhibition of epithelial endometriotic cells.

The dysregulation of apoptosis and in particular apoptosis resistance are other characteristics of endometriosis [24]. Apoptosis (also referred to as programmed cell death) is

important in the control of cell homeostasis in many organisms and is a crucial process in response to cellular stress [25, 26]. Our finding that Cordycepin produced the fragmentation of the PARP-1 precursor into its fragments, an event typical for ongoing apoptosis, clearly demonstrates that 11z epithelial endometriotic cells undergo apoptosis after a 48h treatment with 600  $\mu$ M Cordycepin, after a 72h treatment with 500  $\mu$ M Cordycepin or after a 96h treatment with 400  $\mu$ M Cordycepin. This Cordycepin-induced apoptosis was caspase-dependent, as manifested by the reduction in the levels of the caspase-9 and caspase-3 precursors. Caspases are a family of proteases that are one of the main executors of the apoptotic process. They exist as inactive zymogens and are cleaved to form active enzymes following the induction of apoptosis. The involvement of the caspase-9 indicates that Cordycepin activated the intrinsic apoptotic pathway in these cells, in line with a previous study with mouse leydig tumor cells [27]. Caspase-9 is the initiator caspase of the intrinsic apoptotic pathway. It is activated upon release of cytochrome c from the mitochondria and can activate effector caspases such as caspase-3. This leads to cleavage of key cellular proteins (e.g. PARP-1, cytoskeletal proteins) to drive forward the biochemical events that culminate in death and the dismantling of the cell.

Endometriosis is an estrogen-dependent disease and also depends on multiple growth factors and cytokines [28]. These extracellular growth stimuli are transduced via multiple signaling pathways to intracellular targets that, in turn, control proliferation, growth, and survival. The ERK1/2- and the p38 MAPK-signaling pathways respond to a wide range of stimuli, including stress [29], and transduce extracellular growth stimuli to intracellular targets that, in turn, control proliferation, growth, and survival. Estrogen promotes phosphorylation and activation of ERK1/2 and p38 in endometriotic cells [30, 31] and an inhibitor of p38 suppresses the development of endometriosis in a murine model [18], suggesting that these pathways have a role in endometriosis. We show that Cordycepin abrogated p38

phosphorylation in a time- and concentration-dependent manner in epithelial endometriotic cells, suggesting that Cordycepin downregulates p38-signaling. This reduction, like that of cyclin D1 expression and that of pRb phosphorylation, occurred at non-apoptotic concentrations and thus was not a consequence of apoptosis. However, ERK1/2 signaling was not affected by Cordycepin; the reduction of ERK1/2 phosphorylation detected after a 96h treatment with 600  $\mu$ M Cordycepin is likely a consequence of apoptosis rather than an effect of Cordycepin itself.

The present study demonstrates that Cordycepin suppresses proliferation and survival of epithelial endometriotic cells. Increased proliferation and reduced apoptosis are hallmarks of endometriosis, and Cordycepin may therefore be a potential candidate to therapeutically encounter endometriosis. This is the first study addressing the issue of endometriosis and mRNA polyadenylation, but the intervention at the mRNA processing level presents a novel approach to target endometriotic cells. This may open up research into a potential alternative to current treatment options against endometriosis. Of course, further studies are needed to address the issues of cell-specificity of Cordycepin (endometriotic cells versus normal cells) and of how to administer Cordycepin in endometriosis patients. A possibility may be Cordycepin-encapsulating liposomes, in analogy to ATP-loaded liposomes for treatment of myocardial ischemia [32].

## **Acknowledgements**

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## FIGURE LEGENDS

**Fig. 1.** Cordycepin-induced proliferation inhibition of 11z human epithelial endometriotic cells. Cells were incubated in absence (control) or presence of Cordycepin (250  $\mu$ M, 500  $\mu$ M, 1000  $\mu$ M, 2500  $\mu$ M) for 96h. MTT-assay data are presented as the relative proliferation (expressed as the percentage of untreated control calculated from the respective OD values) as a function of Cordycepin concentration. Data points are the mean  $\pm$  SD of three independent experiments performed in triplicates. \* $p$ <0.05; \*\* $p$ <0.005.

**Fig. 2.** Cordycepin-induced induction of p21 protein expression (**A**) and downregulation of cyclin D1 (**B**) and phosphorylation of pRb at serines 807 and 811 (**C**) in 11z human epithelial endometriotic cells. Cells were incubated without (control) or with different concentrations of Cordycepin for the periods of time indicated and lysed. Proteins were separated by PAGE and blotted. The respective complexes were detected by chemiluminescence and autoradiography.  $\beta$ -Actin was the sample loading control (representative of two independent data sets).

**Fig. 3.** (**A**) Effects of Cordycepin on the phosphorylation of p38 MAPK (threonine 180 and tyrosine 182) and ERK1/2 (threonine 202 and tyrosine 204) assessed by immunoblot analysis. Cells were incubated in absence (control) or in presence of various concentrations of Cordycepin for different periods of time as indicated. Then cells were lysed, proteins were separated by PAGE analysis and blotted, and the respective complexes were detected by chemiluminescence and autoradiography.  $\beta$ -Actin was the sample loading control (representative of two independent data sets). (**B**) A quantitative (based on densitometric analysis) presentation of the Cordycepin-induced changes in phosphorylation of p38 MAPK is given in the bar diagram. The respective data are presented as the relative amount of

phosphorylated p38 to the total amount (phosphorylated and non-phosphorylated) of p38 as a function of Cordycepin concentration and time of treatment.

**Fig. 4.** Cordycepin-induced apoptosis in 11z human epithelial endometriotic cells, presented as the cleavage of the full-length 116 kD PARP-1 precursor into its 89 kD fragment and as the decrease in the levels of the 47 kD caspase-9 and the 37 kD caspase-3 precursors (the respective cleaved fragments were not detected). Cells were incubated in absence (control) or in presence of various concentrations of Cordycepin for different periods of time as indicated. Cells were lysed, proteins were separated by PAGE analysis and blotted, and the respective complexes were detected by chemiluminescence and autoradiography.  $\beta$ -Actin was the sample loading control (representative of two independent data sets).

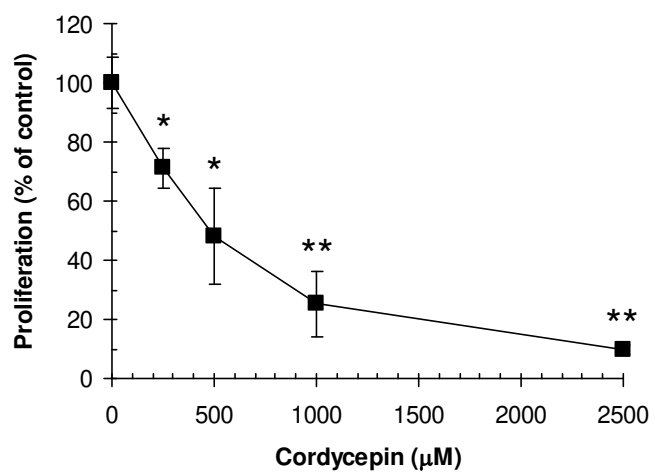


Figure 1

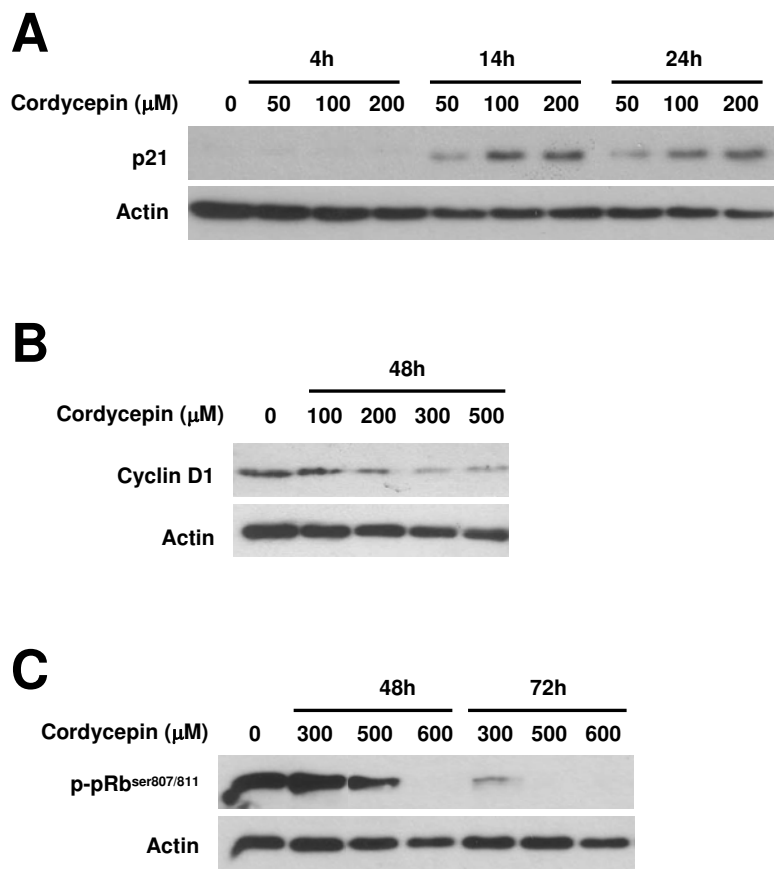
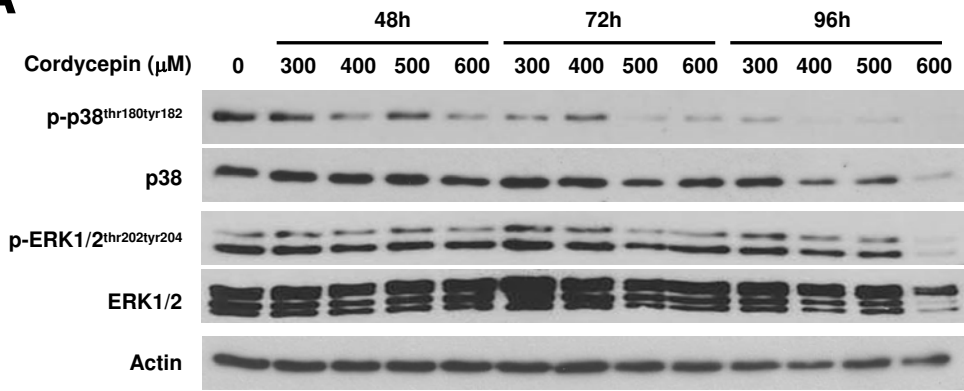


Figure 2

**A**



**B**

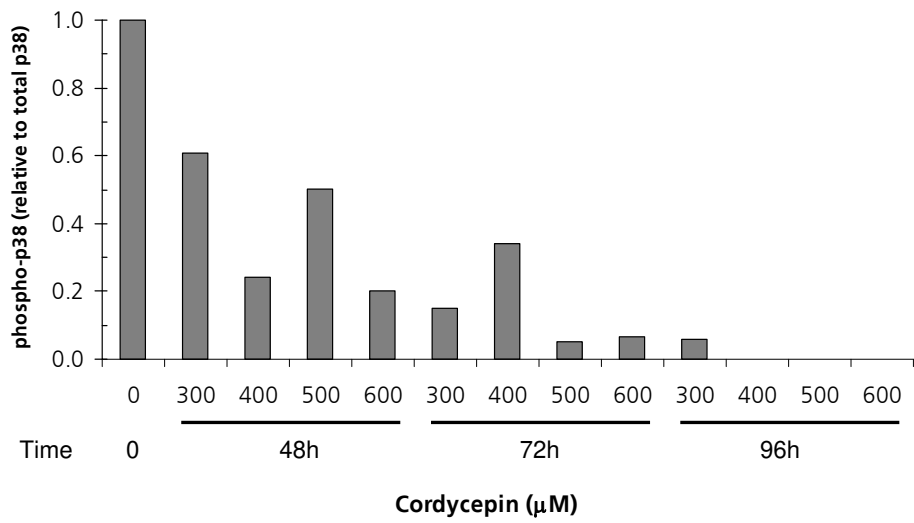


Figure 3

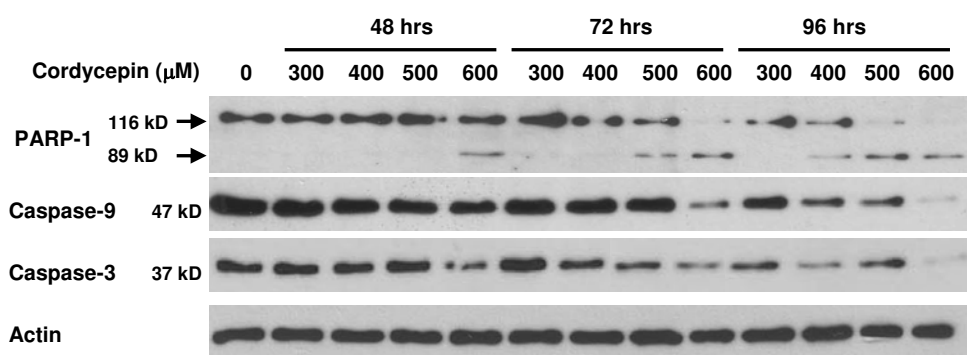


Figure 4